"Adenylate cyclase in the treatment and/or prophylaxis of immune-medicated disease."

Introduction

The invention relates to adenylate cyclase toxin (CyaA) or a derivative or mutant or fragment or variant or peptide thereof.

Adenylate cyclase toxin (CyaA) is a virulence factor of the Gram-negative bacteria, *B. pertussis*, that causes the respiratory disease whooping cough. Bacteria deficient in CyaA are less pathogenic in mice and CyaA has been shown to subvert immune responses to *B. pertussis* by interfering with chemotaxis, phagocytosis and superoxide production in host cells, through the generation of supraphysiological levels of cAMP. Furthermore, CyaA causes lysis and cytotoxicity in a variety of cells and causes apoptosis in macrophages. CyaA is encoded by the *cyaA* gene and is post-translationally activated through palmitoylation of K_{983} by the product of the *cyaC* gene. The C-terminal 1306 amino acids contain a series of nonapeptide repeats involved in calcium binding similar to the repeat in the toxin (RTX) family of exotoxins which have haemolytic and immune stimulatory ability. Acylation or palmitoylation of CyaA by its accessory protein, CyaC, is necessary for binding to and haemolysis of sheep red blood cells and for its ability to lyse macrophages and T cells (1, 2). The N-terminal 400 amino acids contain the catalytic domain that converts ATP to cAMP. Upon cell binding the enzymatic domain is delivered into the cytosol where it must bind eukaryotic calmodulin to become enzymatically active.

The invasive nature of CyaA has been employed to deliver antigenic peptides to the endogenous route of antigen processing for presentation to MHC class I-restricted $CD8^+$ T cells (3). Recently it has been shown that an enzymatically inactive CyaA could deliver an epitope into the MHC class II processing pathway for activation of $CD4^+$ cells (4). In addition, CyaA has been shown to enhance antibody levels to co-administered ovalbumin (5). This study also suggested that a non-active form of

CyaA expressed in *E. coli* in the absence of the *cyaC* gene, which was non-invasive and lacked haemolytic and cytotoxic activity, had limited adjuvant activity for antibody response, when compared with the active toxin (5). CyaA has also been shown to promote Th1 responses to an expressed viral epitope (6). The adjuvant activity of CyaA may reside in its ability to activate cells of the innate immune system through the upregulation of cAMP (7) and/or the binding to the CD11b/CD18 $\alpha_M\beta_2$ integrin (8), expressed on innate immune cells, including macrophages and dendritic cells (DC).

Cells of the innate immune system, especially DC, direct the differentiation of naïve $CD4^+$ T cells into functionally distinct Th1, Th2 or regulatory T (Tr) cell subtypes. Activation of immature DC through binding of conserved microbial molecules to pathogen recognition receptors (PRRs), such as Toll-like receptors (TLR) and integrins, is accompanied by maturation and homing to the lymph nodes, where the mature DC presents antigen to the naïve T cells. Activation of DC by pathogen derived molecules plays a critical role in regulating the differentiation of naïve $CD4^+$ T cells into distinct T cell subtypes (10, 11, 12). Th1 cells confer protection against intracellular infection but are also associated with inflammatory responses and autoimmune disease, whereas Th2 cells are involved in allergic responses. Tr cells are capable of suppressing Th1 and Th2 responses (10, 11, 12).

It is clear that any method of modulating inflammatory activity or inducing regulatory T cells in vivo would be of valuable therapeutic benefit.

Statements of Invention

The invention provides a method for the treatment and/or prophylaxis of an inflammatory and/or immune-mediated disorder comprising the step of administering an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

The invention also provides a method for the treatment and/or prophylaxis of an immune-mediated disorder comprising the step of administering an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

The invention provides a method for the treatment and/or prophylaxis of an autoimmune disease comprising the step of administering an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

The invention also provides use of an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof for the treatment and/or prophylaxis of an inflammatory and/or immune-mediated disorder.

The invention further provides use of an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof for the treatment and/or prophylaxis of an immune-mediated disorder.

The invention also provides use of an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof for the treatment and/or prophylaxis of an autoimmune disease.

In one embodiment the agent may comprise adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof or a product of cells activated by these materials.

In one embodiment of the invention the adenylate cyclase toxin (CyaA) is combined with self or foreign antigens or fragments or mutants or variants or peptides thereof.

In one embodiment the self antigen is selected from any one or more of glutamic acid decarboxylase 65 (GAD 65), myelin oligodendrocyte glycoprotein (MOG), native DNA, myelin basic protein, myelin proteolipid protein, acetylcholine receptor components, thyroglobulin, thyroid stimulating hormone (TSH) receptor, Japanese cedar pollen antigens, ragweed pollen antigens, rye grass pollen antigens, dust mite antigens and feline antigen for animals, histocompatiblity antigens, antigens involved in graft rejection and an altered peptide ligand The antigens involved in graft rejection comprise antigenic components of the graft to be transplanted into the heart, lung, liver, pancreas, kidney of the graft recipient and neural graft components. In a preferred embodiment the self antigen is selected from any one or more of a myelin protein, beta amyloid protein, amyloid precursor protein and collagen and peptides and fragments thereof. The myelin protein may be myelin basic protein or peptides thereof. Preferably the myelin basic protein is myelin oligodendrocyte glycoprotein (MOG) synthetic peptide or fragment or mutant or variant thereof. Most preferably the myelin basic protein is MOG peptide (35-55).

In one embodiment the adenylate cyclase toxin (CyaA) is derived from *Bordetella pertussis*, *Bordetella bronchisepetica* or *Bordetella parapertussis* or related molecules from other bacteria. Related molecules may include proteins from other bacterial with sequences homologous to those in CyaA.

In one embodiment of the invention the agent modulates inflammatory cytokine production.

In one embodiment of the invention the immunomodulatory effects of CyaA on cells of the innate immune system is dependent on co-activation with a Toll-like receptor ligand, such as LPS or other toll-like receptor ligands, selected from any one or more of CpG motifs, dsRNA, Poly (I:C) and the lipopeptide Pam3Cys.

In one embodiment of the invention the CyaA promotes IL-10 and IL-6 production by macrophages and dendritic cells.

In another embodiment of the invention CyaA synergises with LPS to promote IL-10 and IL-6 production by macrophages and dendritic cells.

In one embodiment of the invention the CyaA inhibits inflammatory cytokines, chemokines or other inflammatory mediators. The inflammatory cytokine may be selected from any one or more of IL-12 or TNF-α, IFN-γ, IL-1, IL-23 and IL-27. The inflammatory chemokine may be macrophage inflammatory protein-1α or macrophage inflammatory protein-1β.

In one embodiment of the invention the CyaA promotes dendritic cell maturation following co-activation with TLR-ligands. In one case the CyaA promotes CD80 expression by dendritic cells.

In one embodiment of the invention CyaA inhibits TLR-ligand-induced dendritic cell activation. In one case the CyaA inhibits CD40 and ICAM-1 expression.

In one embodiment of the invention CyaA acts as an adjuvant *in vivo* to promote the induction of Th2 or Tr cells to co-administered antigens.

In another embodiment CyaA acts as an adjuvant *in vivo* to promote IgG1 antibodies to co-administered antigens.

The co-administered antigens may comprise self or foreign antigens.

In one embodiment of the invention the CyaA is present in a non-palmitoylated form.

In one embodiment of the invention the CyaA is substantially endotoxin free. The CyaA may comprise less than 300 pg endotoxin / μg protein.

In another embodiment of the invention the CyaA is in the form of an immunomodulator, adjuvant, immunotherapeutic or anti-inflammatory agent.

In one embodiment of the invention the agent modulates inflammatory cytokine production induced by infection or trauma.

In one embodiment of the invention the disorder is sepsis or acute inflammation induced by infection, trauma or injury.

In another embodiment of the invention the disorder is selected from any one or more of Crohn's disease, inflammatory bowel disease, multiple sclerosis, type 1 diabetes, rheumatoid arthritis and psoriasis. Other immune-mediated disorders include any one or more of diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), Sjogren's Syndrome, including keratoconjunctivitis sicca secondary to Sjogren's Syndrome, alopecia areata, allergic responses due to arthropod bite reactions, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scieroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, uveitis posterior, interstitial lung fibrosis, Alzheimers disease or coeliac disease.

In a further embodiment of the invention the disorder is asthma or atopic disease.

In one embodiment of the invention the agent is in a form for oral, intranasal, intravenous, intradermal, subcutaneous or intramuscular administration. The agent may be administered repeatedly.

The invention also provides a product comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof. The product may be in combination with an antigen wherein said antigen is a self or foreign antigen. The CyaA may comprise a derivative or mutant or fragment or variant or peptide thereof or a product of cells activated by these materials.

The invention also provides a pharmaceutical composition comprising CyaA or derivative or mutant or fragment or variant or peptide thereof.

The invention further provides a pharmaceutical composition comprising CyaA or derivative or mutant or fragment or variant or peptide thereof as adjuvant for immunization with a self or foreign antigen.

The invention further provides a pharmaceutical composition comprising CyaA or derivative or mutant or fragment or variant or peptide thereof in combination with an antigen, where said antigen is selected from a self-antigen and a foreign antigen.

In one embodiment the CyaA comprises a derivative or mutant or fragment or variant or peptide thereof or a product of cells activated by these materials.

In one embodiment the self antigen is selected from any one or more of glutamic acid decarboxylase 65 (GAD 65), native DNA, myelin basic protein, myelin proteolipid protein, acetylcholine receptor components, thyroglobulin, thyroid stimulating hormone (TSH) receptor, Japanese cedar pollen antigens, ragweed pollen antigens, rye grass pollen antigens, and dust mite antigens and feline antigens, histocompatibility antigens, antigens involved in graft rejection and an altered peptide ligand.

The antigens involved in graft rejection may include antigenic components of the graft to be transplanted into the heart, lung, liver, pancreas, kidney for graft recipient and neural graft components.

The composition may comprise non-acylated CyaA or derivative or mutant or fragment or variant or peptide thereof.

The invention also provides an immunomodulator comprising adenylate cyclase toxin (CyaA).

The invention further provides a recombinant non-acylated CyaA having immunomodulatory effects.

The invention also provides a vaccine comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof. The vaccine may comprise an antigen.

In one embodiment of the invention the CyaA and antigen are present in a by weight ratio range of 0.01:1 to 100:1.

In another embodiment of the invention the CyaA and antigen are present in a molar ratio of 1:10 to 10:1.

The invention also provides antibodies to adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide thereof.

The invention also provides an amino acid sequence selected from any one or more of SEQ ID No. 3 or 4.

The invention further provides use of an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide or product of cells activated by the agent for the treatment and/or prophylaxis of an inflammatory and/or immune-mediated disorder.

The invention provides use of an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or variant or peptide or product of cells activated by the agent for the prophylaxis and/or treatment of diseases or conditions involving Toll-like receptor (TLR) dependent signalling.

The invention further provides use of an agent comprising adenylate cyclase toxin (CyaA) or derivative or mutant or fragment or variant or peptide or product of cells activated by the agent for the prophylaxis and/or treatment of asthma or allergy.

The term derivative or mutant or fragment or variant or peptide as used herein are understood to include any molecule or macromolecule consisting of a non-acylated or non-palmitoylated derivative of CyaA or a functional portion of acylated or non-acylated CyaA. Fragments or variants or peptides may be prepared by techniques commonly known to the skilled person in the art. These include peptides or fragments corresponding to the regions of CyaA that interact with CD11b/CD18.

The term antigen refers to a molecule which can initiate a humoral and/or cellular immune response in a recipient of the antigen. The term antigen is taken to mean any substance that is recognized by an antibody or T cell receptor. The term self- or auto-antigen is taken to mean an endogenous antigen on a self-tissue or cell in the body, which is not foreign. The term foreign antigen is taken to mean an antigen from a pathogen (bacteria, virus, fungi or parasite).

Antigens involved in autoimmune diseases, allergy, and graft rejection can be used in the compositions and methods of the invention. Examples of antigens involved in autoimmune disease include myelin oligodendrocyte glycoprotein (MOG) glutamic

acid decarboxylase 65 (GAD 65), native DNA, myelin basic protein, myelin proteolipid protein, acetylcholine receptor components, thyroglobulin, and the thyroid stimulating hormone (TSH) receptor. Examples of antigens involved in allergy include pollen antigens such as Japanese cedar pollen antigens, ragweed pollen antigens, rye grass pollen antigens, animal derived antigens such as dust mite antigens and feline antigens or histocompatiblity antigens. Examples of antigens involved in graft rejection include antigenic components of the graft to be transplanted into the graft recipient such as heart, lung, liver, pancreas, kidney, and neural graft components. An antigen can also be an altered peptide ligand useful in treating an autoimmune disease. Examples of miscellaneous antigens which can be can be used in the compositions and methods of the invention include beta amyloid protein or amyloid precursor protein.

The term adjuvant is taken to include a substance used in conjunction with an antigen to enhance the immune response to the antigen in vivo.

The term immunomodulator is taken to include any molecule, including those derived from bacteria, viruses, parasites or fungi pathogens, that modulates ie increases and/or decreases the responses of cells of the immune system.

Brief Description of the Invention

The invention will be more clearly understood from the following description thereof with reference to the accompanying drawings in which: -

Fig. 1 are graphs showing the levels of IFN-γ, IL-4, IL-5 and IL-10 produced by lymph node cells from mice after immunization with PBS, KLH alone or with CyaA. BALB/c mice were immunized s.c. in the hind footpad with PBS, KLH (5 μg) alone or with CyaA (1 μg). After 7 days mice were sacrificed and popliteal lymph node cells prepared and stimulated with KLH (2-50 μg/ml) or medium only. After 3 days supernatants were tested for IFN-

γ, IL-4, IL-5 and IL-10 by ELISA. Proliferation was assayed on day 4 by ^{3}H-Thymidine incorporation. Results represent means (+ SD) of 5 mice per group and are representative of 3 experiments. *, $P < 0.05$; ***, $P < 0.001$ KLH versus KLH + CyaA;

Fig. 2 are graphs showing IL-4, IL-5, IL-10 and IFN-γ production by T cell lines and clones generated from mice immunized with KLH in the presence of CyaA. A) $CD4^{+}$ T cell lines were generated from lymph nodes of 10 individual mice immunized with KLH and CyaA. B) T cell line 7.2 was cloned by limiting dilution. T cell lines or clones were stimulated with KLH (50 μg/ml) in the presence of autologous APC and cytokine concentrations tested in the supernatants after 3 days, IFN-γ was at background levels in each of the T cell clones;

Fig. 3 is a graph showing antigen-specific IgG, IgG1 and IgG2a levels in mice after immunisation s.c. in the hind footpad with PBS, KLH (5 μg) alone or with CyaA (1 μg) and boosted 21 days later. Serum samples were taken 7 days after one (A) or two (B) immunizations and KLH-specific IgG, IgG1 and IgG2a titres were determined by ELISA. Results are mean (+ SD) titres for 5 mice per group and are representative of 2 experiments. ***, $P < 0.001$ KLH versus KLH + CyaA;

Fig. 4 are graphs showing IL-10, IL-6 and TNF-α levels produced by macrophages incubated with LPS, CyaA, lipopolysaccharide (LPS) and CyaA or CyaA in the presence or absence of polymyxin B. CyaA enhances LPS-induced anti-inflammatory cytokines and suppresses pro-inflammatory cytokines by macrophages. J774 macrophages ($1x10^{6}$/ml) were incubated with the indicated concentrations of LPS (0-1000 ng/ml), CyaA (1 μg/ml), in presence or absence of polymyxin B (PB; 10 μg/ml). Supernatants were collected at the indicated times and were tested for IL-10, IL-6 and TNF-α by immunoassay. Results are means (+ SD) of triplicate assays and are

representative of 3 experiments. **, $P < 0.01$; ***, $P < 0.001$ versus CyaA; ++, $P < 0.01$, +++, $P < 0.001$; versus LPS alone at the same concentration;

Fig. 5 are graphs showing IL-10, IL-6, TNF-α and IL-12p70 levels in dendritic cells (DC) following incubation with LPS, CyaA LPS and CyaA or CyaA in the presence or absence of polymyxin-B. CyaA enhances LPS-induced anti-inflammatory cytokines and suppresses LPS-induced pro-inflammatory cytokines from DC. Murine bone marrow-derived immature DC ($1x10^6$/ml) were incubated with the indicated concentrations of LPS (0-1000 ng/ml), CyaA (1 μg/ml) in presence or absence of polymyxin-B (PB; 10 μg/ml). Supernatants were collected at the indicated times and tested for IL-10, IL-6, TNF-α and IL-12p70 by immunoassay. Results are means (±SD) of triplicate assays and are representative of 3 experiments. **, $P < 0.01$; ***, $P < 0.001$ versus CyaA; +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.01$ versus LPS alone at the same concentration;

Fig. 6 are immunofluorescence graphs showing the CD80, CD86, MHC-II, CD40 and ICAM-I expression on DC. CyaA enhances CD80, CD86 and MHC-II, but inhibits CD40 and ICAM-I expression on DC. DC were stimulated with CyaA (1 μg/ml) in the presence of polymyxin B (10 μg/ml), LPS (1 μg/ml), CyaA and LPS or medium only. After 24 h incubation, cells were washed and stained with antibodies specific for CD80, CD86, MHC-II, CD-40 and ICAM-I or with isotype matched controls. Results of immunofluorescence analysis are shown for treated (black line) compared to untreated (grey histogram) DC. Profiles are shown for a single experiment and are representative of 2 separate experiments;

Fig. 7 are graphs showing cytokine production by lymph node cells from immunized TLR4-defective mice. Bone marrow derived DC from C3H/HeN or C3H/HeJ mice ($1x10^6$/ml) were cultured with the indicated concentrations of LPS (0-10 ng/ml), CyaA (1 μg/ml), in presence or absence of polymyxin-

B (PB; 10 μg/ml). Supernatants collected and tested by immunoassay for IL-10 and MIP1-α (4 h) and IL-12p70 and TNF-α (24 h). Results are means (±SD) of triplicate assays and are representative of 3 experiments. ++, $P <$ 0.01 versus CyaA; $P < 0.001$ versus LPS alone at the same concentration;

Fig. 8 are immunofluorescence graphs showing that CyaA-induced DC activation is altered in TLR4-defective mice. Bone marrow derived DC from C3H/HeN (A) or C3H/HeJ (B) mice ($1x10^6$/ml) were cultured with CyaA (1 μg/ml) either alone or with polymyxin B (PB; 10 μg/ml) or LPS (10 ng/ml) or with medium. After 24 h incubation, cells were washed and stained with antibodies specific for CD80, CD86, MHC-II, CD-40 and ICAM-I or with isotype matched control antibodies. Immunofluorescence analysis are shown for treated (black line) compared to untreated (grey histogram) DC. The numbers on the right of each histogram refer to the mean florescence intensity of the treated cells, the value for cells treated with medium only is shown on the left of the first histogram in each case. Profiles are shown for a single experiment and are representative of 3 experiments;

Fig. 9 are images of gels showing the palmitoylation status of A-CyaA and NA-CyaA. *E. coli* XL1-blue harbouring the plasmids pJR1 and pJR2, encoding CyaA and CyaA + CyaC respectively, were grown in selective medium supplemented with [^{14}C(U)] palmitic acid. Protein expression was induced with 1 mM IPTG for 15 minutes. Bacteria were pelleted and washed in fresh medium before separation on a 7% SDS-PAGE gel (A) and visualised with gel-code blue reagent before flourographic analysis to visualise the radiolabelled proteins (B). Lane 1, NA-CyaA; lane 2, A-CyaA; lane 3, highly purified recombinant A-CyaA (not radioactively labelled). The position of a 250 kd molecular weight marker is indicated.

Fig. 10 is a graph showing the percentage haemolysis of red blood cells (RBC) under various conditions. 100 μl of $5x10^8$ RBC/ml were treated with

the indicated amounts (μg/ml) of non-acylated (NA-CyaA) or acylated CyaA (A-CyaA) for 16 h. 50 l of supernatant was collected and the absorbance at OD_{541} measured and used to calculate the percentage haemolysis. Results were compared by the one-way ANOVA with Tukey post-test. $^{**}P<0.01$: treated sample versus medium. Results are means ± SD of a representative experiment performed three times in triplicate;

Fig. 11 are a graphs showing that induction of cell lysis and caspase-3 activation is associated with adenylate cyclase activity and intracellular cAMP elevation, but is not dependent on acylation. (A) Cell free adenylate cyclase activity of CyaA or CyaA-derivatives. (B) Intracellular cAMP accumulation in J774 macrophages in response to CyaA or CyaA-derivatives (10 g/ml). (C) Lysis of J774 cells in response to 10 g/m l CyaA or CyaA derivatives determined by LDH release assay. (D) Caspase-3 activation induced by CyaA or CyaA derivatives (10 g/ml) in J774 macrophages expressed as relative fluorescence units (RFU) over time. Results are representative of experiments repeated at least twice. * $P<0.05$, ** $P<0.01$ versus medium control;

Fig 12 are graphs showing the influence of toxin concentration on intracellular cAMP accumulation, caspase-3 activation and cell lysis induced by acylated and non-acylated CyaA. (A) Intracellular cAMP accumulation in J774 macrophages in response to increasing concentrations of A-CyaA or NA-CyaA. (B) Caspase-3 activation induced by A-CyaA or NA-CyaA (0.3-10 g/ml) in J774 macrophages, expressed as relative fluorescence units (RFU) over time. (C) Lysis of J774 cells, determined by LDH release assay, in response to A-CyaA, NA-CyaA (0.3-10 g/ml), LPS (0.2 μg/ml) or medium (Med) only in the absence (C) or presence (D) of polymyxin B. Results are representative of experiments repeated at least twice. *** $P<0.001$, versus medium control;

Fig. 13 are graphs showing that acylation is necessary for CyaA-induced morphological changes in macrophages. J774 macrophages were untreated (A), or treated with 3 g/ml (B), 5 g/ml (C) or 10 g/m l (D) of A-CyaA or 1 M cycloheximide (E) or 3 g/ml (F), 5 g/ml (G) or 10 g/m l (H) NA-CyaA for 6 h. Altered morphology after treatment with A-CyaA and cycloheximide compared with normal morphology in untreated and NA-CyaA treated cells;

Fig. 14 are graphs showing that acylation of CyaA is not required for the modulation of cytokine production by macrophages. J774 macrophages were incubated with A-CyaA or NA-CyaA (1 g/ml) in the presence or absence of 10 g/ml polymyxin B. 2 h later 10 ng/ml LPS was added as indicated on the figure. After a further 4 h incubation, supernatants were collected and IL-10 and TNF- concentrations determined by ELISA. Results were compared by the one-way ANOVA with Tukey post-test. *** $P<0.001$: CyaA and LPS versus LPS. Results are means ± SD of triplicate assays and are representative of 3 experiments;.

Fig. 15 are graphs showing the modulation of macrophage cytokine production by A-CyaA and NA-CyaA at different concentrations. J774 macrophages were treated with A-CyaA and NA-CyaA at the indicated concentrations (g/ml), with and without the addition of 10 ng/ml LPS 2 hours later. After a further 4 h incubation, supernatants were collected and IL-10 and TNF- concentrations determined by ELISA. Results were compared by the one-way ANOVA with Tukey post-test. *$P<0.05$, **$P<0.01$, *** $P<0.001$: CyaA and LPS versus LPS. +$P<0.05$, ++$P<0.01$, +++$P<0.001$: A-CyaA versus NA-CyaA at same concentration. Results are means ± SD of a representative experiment performed twice in triplicate;

Fig. 16 are graphs showing that non-acylated CyaA modulates CpG-ODN-stimulated cytokine and chemokine release from DC. DC from C3H/HeJ

mice were incubated with 1 g/ml A-CyaA or NA-CyaA for 2h, before the addition of 10 g/ml CpG-ODN. Supernatants were tested for the presence of IL-10, TNF- and CCL3 4h later and for IL-12 p70 24 h later. Results were compared by the one-way ANOVA with Tukey post-test. *P<0.05, **P<0.01, *** P<0.001: CpG-ODN versus CyaA and CpG-ODN. Results are means ± SD of a representative experiment performed twice in triplicate;

Fig. 17 are graphs showing that CyaA-induced modulation of macrophage activation is dependent on interaction with CD11b. J774 macrophages were incubated with 1ug/ml A-CyaA or NA-CyaA for 2h, before the addition of 10 g/ml CpG-ODN. Cells were incubated with 10 g/ml anti-CD11b or isotype control antibody prior to the addition of CyaA. Results were compared by the one-way ANOVA with Tukey post-test. *P<0.05, **P<0.01, *** P<0.001: anti-CD11b versus control antibody. +++ P<0.001: CpG-ODN + toxin versus CpG alone. Results are means ± SD of a representative experiment performed twice in triplicate;

Fig. 18 are graphs showing that acylation is not essential for modulation of DC maturation by CyaA. DC from BALB/c mice were treated with 1 g/m l NA-CyaA or A-CyaA in the presence or absence of 10 g/m l polymyxin B (PB). 2 h later 1 g/ml LPS or medium only was added. 24 h later cells were harvested, labelled with biotin-conjugated hamster anti-CD11c IgG and streptavidin-PerCP or isotype control. Cells were simultaneously stained with either FITC- or phycoerythrin-labelled anti-CD80, anti-CD86, anti-MHC class II, anti-CD40 or anti-ICAM-1 or the appropriate isotype control antibody. Results of immunofluorescence analysis are shown for treated (black lines) compared with untreated (filled black histograms) DC and are representative of 2 experiments;

Fig. 19 are graphs showing that NA-CyaA is as effective an adjuvant as A-CyaA, preferentially enhancing Th2/Tr1 type response *in vivo*. BALB/c mice

were immunised in the footpad with PBS, KLH alone or with NA-CyaA or A-CyaA. 7 days later popliteal lymph node suspensions were prepared and KLH-specific cytokine release (A) and proliferation (B) determined. Serum was tested for KLH-specific IgG, IgG1 and IgG2a by ELISA and expressed as end point titres (C). Results are means ± SD for 5 mice, with samples tested in triplicate, and are representative of two experiments;

Fig. 20 is a graph showing the effect of immunization with myelin oligodendrocyte (MOG) peptides with CyaA on the disease progression in experimental autoimmune encephalomyelitis (EAE), a murine model for multiple sclerosis. Mice were immunized subcutaneously (s.c.) with 50 μg MOG peptide (residues 35-55) and 1.0 μg CyaA in phosphate buffered saline. This was repeated 21 days later. Control mice received MOG peptide or saline only. 7 days after the second immunization, EAE was induced by s.c. administration of 150 μg MOG peptide emulsified in complete Freund's adjuvant, supplemented with 1 mg *Mycobacteria tuberculosis* intraperitoneal (i.p.) injection of 500 ng pertussis toxin, followed 2 days later by a second i.p. injection with 500 ng pertussis toxin. Mice were assessed daily for clinical signs of EAE, and scored as follows: 1 = tail paralysis, 2 = wobbly gait, 3 = hind limb weakness, 4 = hind limb paralysis, 5 = complete paralysis of hind and fore limbs, 6 = death. The disease index was calculated by adding all daily average disease scores, dividing the average day of onset, an multiplying by 100;

Fig. 21 is a graph showing the effect of immunization with myelin oligodendrocyte (MOG) peptides with CyaA on EAE average disease score over time;

Fig. 22 are histopathology sections of spinal cords of mice after induction of EAE (untreated) or after immunization with myelin oligodendrocyte peptide (MOG) or MOG peptide + CyaA (MOG + CyaA). EAE was induced and

mice immunized as described in Fig. 16, sections of spinal cord were removed from mice 19-23 days after induction of EAE and stained with haematoylin and eosin. The EAE induction in un-treated and MOG-immunized mice is severe with a pronounced mononuclear cell infiltrate; this is considerable reduced in mice immunized with MOG and CyaA;and

Fig. 23 is a plasmid expression vector for pNM2 (pQE80+TMCyaA+CyaC;

Fig. 24 is a plasmid expression vector for pJR2 (pQE80+CyaA+CyaC);

Fig. 25 is a plasmid expression vector for pJR1 (pQE80+CyaA); and

Fig. 26 is a plasmid expression vector for pAPB22 (pQE80+TMCyaA).

Detailed description

We have found that adenylate cyclase toxin (CyaA) or a non-acylated derivative of CyaA (NA-CyaA) from *Bordetella pertussis* in combination with a Toll-like receptor (TLR) ligand promotes the induction of interleukin (IL)-10 and IL-6 production by cells of the innate immune system. Furthermore we have found that CyaA and NA-CyaA promotes the induction of anti-inflammatory cytokines or regulatory T (Tr) cells to co-administered antigens. The induction of Tr cells *in vivo* has potential for the treatment of inflammatory or autoimmune disease or allergy.

We have also found that acylation of the toxin is necessary for cytotoxicity, but not for immunomodulation. A non-acylated or non-palmitoylated CyaA molecule had reduced cyotoxicity, but retained its immunomodulatory function. Therefore *Bordetella pertussis* adenylate cyclase toxin or derivatives or mutant or fragment or variant or peptide thereof or a product of cells activated by these materials have valuable potential as immunomodulators, adjuvants, immunotherapeutics or anti-inflammatory agents. Compositions comprising non-acylated CyaA would be

particularly valuable as an immunomodulators, adjuvants, immunotherapeutics or anti-inflammatory agents.

CyaA can subvert host immune responses and thereby contribute to colonisation and persistence of *B. pertussis* in the respiratory tract. Previous investigations have demonstrated adjuvant activity for the wildtype toxin, but the interpretation of these findings was complicated by the presence of relatively high concentrations of lipopolysaccharide (LPS), known to be closely associated with purified CyaA. We examined immunomodulatory properties of CyaA and the possible contribution of LPS, known to be present in purified CyaA preparations.

CyaA enhanced IL-5 and IL-10 production and IgG1 antibodies to co-administered antigen *in vivo*. Antigen-specific $CD4^+$ T cell clones generated from immunised mice had cytokine profiles characteristic of Th2 and type 1 Tr (Tr1) cells. Since innate immune cells direct the induction of T cell subtypes, we examined the influence of CyaA on activation of dendritic cells (DC) and macrophages. CyaA significantly augmented LPS-induced IL-6 and IL-10 and inhibited LPS driven TNF-α and IL-12p70 production from bone marrow-derived DC and macrophages. CyaA also enhanced cell surface expression of CD80, CD86 and MHC class II on immature DC. The stimulatory activity of the CyaA preparation for IL-10 production and CD80, CD86 and MHC class II expression was attenuated following addition of polymyxin B or with DC from TLR 4-defective mice. Treatment of DC with LPS alone at a concentration present in the CyaA preparation (0.2 ng/ml) failed to activate DC *in vitro*. We have found that activation of innate cells in vitro by CyaA is dependant on a second signal through TLR4 and that CyaA can promote Th2/Tr1 cell responses by inhibiting IL-12 and promoting IL-10 production by DC and macrophages.

As CyaA causes cytotoxicity of mammalian cells, it may not be suitable for clinical use in humans. However we have prepared non-toxic derivatives and mutants that retain immunomodulatory activity.

CyaA belongs to the RTX family of toxins, which require post translational acylation for activation. Acylation or palmitoylation of CyaA is necessary for the toxin to lyse macrophages. Acylated (A-CyaA) and non-acylated CyaA (NA-CyaA) molecules were expressed in *E. Coli* and examined for cytotoxicity and immunomodulatory function. Both proteins enhanced LPS driven IL-10 and IL-6 production and inhibited LPS-stimulated IL-12, TNF and the chemokine CCL3 (MIP-1) production in macrophages and DC. At low doses acylated CyaA is more efficient than nonacylated CyaA at eliciting these effects. Both NA-CyaA and A-CyaA also modulated CpG (TLR-9 ligand) and poly(I:C) (TLR3 ligand) as well as LPS-driven cytokine production in DC and macrophages. In addition, despite the lack of acylation, CyaA stimulated cAMP accumulation in macrophages and DC. Both proteins stimulated DC maturation leading to increased surface expression of CD80 and MHC-II and decreased expression of LPS-stimulated CD86, CD40 and ICAM-1. The non-acylated CyaA was unable to lyse macrophages or red blood cells even at doses 10 fold higher than that which elicited immunomodulation. Both proteins have similar adjuvant activity *in vivo*, inducing IgG1 antibody and Th2 and Tr cells specific for co-administered antigens. These results indicate that the recombinant non-acylated CyaA molecule lacks cytotoxicity but retains its immunomodulatory effects.

Unlike acylation, enzyme activity was essential for the immunomodulatory activity of CyaA. Regardless of their acylation status, CyaA mutants lacking functional adenylate cyclase activity were unable to cause an increase of intracellular cAMP concentrations and were unable to suppress LPS-induced TNF- release or to enhance IL-10 production by innate immune cells. This suggests that the increase in the concentration of intracellular cAMP is one signal responsible for modulation of innate immune cell activation by CyaA.

CyaA is a member of the RTX family of pore-forming proteins, but it has a unique bifunctional nature, with an enzymatic domain located in the NH_2-terminal and the

RTX domain in the COOH-terminal. This domain arrangement allowed the examination of the separate activities of CyaA-cell lysis, cAMP accumulation, immunomodulation, apoptosis, in a way that is not possible with any other RTX toxin. Lysis of eukaryotic cells by RTX toxins is dependent on post-translational acylation. It has been reported by others that acylation of the RTX domain of CyaA is necessary for its haemolytic and cytotoxic activity (1, 2, 13).

We have found that acylation is not essential for CyaA to induce cAMP accumulation in macrophages or to modulate activation of macrophages and DC. Like A-CyaA, NA-CyaA synergised with LPS to enhance secretion of IL-10, while inhibiting TNF- and IL-12 p70 production. The modulatory effects of A-CyaA and NA-CyaA on innate cell cytokine production were not confined to the TLR4 ligand, LPS, which is closely associated with CyaA, but were also observed with the TLR9 ligand, CpG-ODN. NA-CyaA and A-CyaA had similar effects on DC maturation, upregulating CD80 expression, while downregulating CD40 and ICAM-1 expression. Furthermore NA-CyaA, like A-CyaA, was an effective adjuvant *in vivo* when co-injected with a foreign antigen, stimulating the production of antigen-specific IgG1 antibodies and IL-4, IL-5 and IL-10 production by T-cells.

RTX toxins bind to leucocytes through β_2 integrins and CD11b/CD18 has been identified as the receptor for CyaA (14, 13) In order to bind red blood cells, which lack β_2 integrins, CyaA and other RTX proteins require acylation (1).

We have found that acylation may influence, but is not essential for CyaA to induce cAMP accumulation in innate immune cells expressing CD11b/CD18. Previous studies had suggested that acylation of CyaA was necessary for the induction of increased cAMP levels in eukaryotic cells. However, these studies did not use innate immune cells but rather red blood cells and Jurkat T cells, each of which express low or no CD11b/CD18, and CHO cells transfected with CD11b (13, 1). The CD11b binding site on CyaA has been localised to a glycine/aspartate-rich region between residues 1166 and 1281 of the RTX domain (13). CyaA proteins containing

mutations within this region bind red blood cells, but not CD11b-expressing cells. Conversely, a CyaA protein with a FLAG epitope insert at amino acid 926 bound CD11b-expressing cells, but not red blood cells. The authors suggested that this was due to the disruption of the structure required for acylation of the adjoining Lys^{983}.

We found that acylation is not required for CyaA-induced cAMP accumulation and immunomodulation in macrophages and that anti-CD11b antibodies block the modulatory effects of A-CyaA and NA-CyaA. This indicates that posttranslational modification is not essential for interaction of CyaA with its β_2 integrin receptor CD11b/CD18 on the host cell surface. Interaction of CyaA with CD11b/ CD18, which is primarily expressed on macrophage and myeloid DC, may be a strategy evolved by the bacteria to target and disrupt cytokine signalling pathways in key cells of the innate immune system.

It is likely that the acyl groups also associate with the eukaryotic cell membrane increasing the efficiency of CyaA-host cell interactions and facilitating the transmembrane delivery of the adenylate cyclase domain into target cells. This interaction may be of increased importance for cells lacking CD11b/CD18 and may explain the failure of NA-CyaA to deliver the adenylate cyclase domain into red blood cells, Jurkat T cells and CD11b transfected CHO cells. The increase in intracellular cAMP caused by the cytosolic adenylate cyclase domain leads to modulation of host cell function, including altered cytokine and chemokine release by macrophages and DC. CyaA oligomerises in the cell membrane to form pores and thus cause membrane disintegration. This indicates that in cells expressing the CyaA receptor CD11b/CD18, such as DC and macrophages, acylation is important for cytotoxicity.

CyaA induction of cell death in macrophages is indicated by the DNA fragmentation seen in CyaA-treated cells *in vitro* (2). CyaA-deficient *B. pertussis* does not induce lysis of J774 macrophages, has reduced capacity to cause apoptosis of alveolar macrophages *in vivo* and is considerably less virulent in mice (2, 15). We observed

cell death in macrophages incubated with A-CyaA or an enzyme inactive A-CyaA. In contrast cell lysis was only observed with the highest dose of NA-CyaA examined and this effect was abolished, as was a proportion of the lytic activity of A-CyaA by co-incubation with polymyxin B. LPS and LPS-induced TNF-α have been associated with apoptosis (16) and may therefore have contributed to the cell death observed.

We have found that CyaA also induces activation of caspase-3, a key effector molecule in apoptosis. Caspase-3 activation was observed in macrophages treated with the wildtype toxin (A-CyaA) and the enzymatically inactive mutant (A-iAC-CyaA), but not with NA-CyaA. NA-iAC-CyaA at high concentrations (10μg/ml) did activate caspase-3. Previously it has been demonstrated that cAMP-induction is associated with transient inhibition of caspase-3 activation and apoptosis (17, 18). Therefore the induction of caspase-3 by NA-iAC-CyaA may reflect removal of the inhibitory influence of cAMP induction, thereby decreasing the threshold for caspase-3 activation seen with the NA-CyaA. It appears that acylation, but not adenylate cyclase activity, is required for CyaA-induced cell death.

We have found that acylation of RTX domains is important in the immunomodulatory and pro-apoptotic activities of bacterial toxins. The non-acylated enzymatially active toxin retains the ability to enhance cAMP, albeit at a slightly lower efficiency than the wildtype toxin. Elevation of intracellular cAMP appears to be a critical factor in the immunomodulatory activity of CyaA, but is not essential for its ability to cause cell lysis or caspase-3 activation and may even inhibit these effects. Like the wild-type toxin, this non-acylated derivative specifically targets CD11b/CD18 expressing macrophages and DC, inhibiting the release of pro-inflammatory cytokines and enhancing IL-10 production and promoting maturation of DC into a phenotype that directs the induction of Th2 and IL-10-producing Tr1 cells. Thus, NA-CyaA is capable of promoting both innate and adaptive IL-10 production and as a consequence has considerable potential as an adjuvant or immunotherapeutic agent for the prevention and/or treatment of inflammatory and Th1-mediated autoimmune diseases.

Acylated and non-acylated CyaA or derivatives or mutants or variants or peptides thereof may be used as immunomodulators and therapeutics for the treatment of inflammation or immune mediated diseases. Inflammation and hyperactive T cell responses are features of a number of immune mediated diseases. Autoimmune diseases including multiple sclerosis, rheumatoid arthritis, type 1 diabetes and Crohn's disease involve T cells that secrete interferon (IFN)-γ, termed type 1 T helper (Th1) cells, and inflammatory responses against self-antigens. In contrast atopic diseases and asthma are mediated by the reciprocal Th2 subtype of T cells.

Many of the diseases detailed above have no satisfactory treatment. Traditional therapies for inflammatory and immune-mediated diseases have largely relied on steroids and non-steroidal anti-inflammatory drugs, however, these are non-specific and have side effects. More recently drugs that inhibit key inflammatory cytokines, in particular tumour necrosis factor (TNF)-α, have been developed. These include antibodies or soluble TNF receptors that are effective against certain autoimmune diseases, but are associated with side effects (including recurrent tuberculosis) and are limited to diseases where TNF-α is the key mediator of pathology. Another therapeutic approach is the direct administration of anti-inflammatory cytokines (e.g. IL-10), but this is compromised by the short half-life of the cytokines *in vivo*.

An alternative strategy is to employ agents that induce anti-inflammatory cytokines, such as IL-10, which will have a direct immunosuppressive effect *in vivo* and will also, in the presence of antigen, prime IL-10 secreting antigen-specific Tr cells, which will amplify IL-10 production and the immunosuppressive effect.

We have found that CyaA and derivatives thereof have the potential to drive innate and adaptive IL-10 and thereby act as anti-inflammatory agents and either as immunotherapeutics or as components of vaccines to prevent immune mediated disease. We have shown that CyaA can reduce the severity of disease in experimental autoimmune encephalomyelitis (EAE), a murine model of multiple

sclerosis (MS). Immunization of mice with MOG peptide in the presence of CyaA delayed the development of EAE and reduced the incidence of disease in EAE, a murine model for multiple sclerosis. CyaA and the non-toxic NP-CyaA have considerable potential as anti-inflammatory agents, immunotherapeutics and immunomodulators and adjuvants for vaccines in the prevention of inflammatory or autoimmune diseases.

The invention will be more clearly understood from the following examples thereof.

Examples

A protein upon which this work is based is the CyaA protein of *Bordetella pertussis* (21). The sequence of the wildtype protein can be found in the online database of the NCBI here:

http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=33591934

Plasmid construction. Genomic DNA of *B. pertussis* (strain W28) was prepared from a mid-log culture. The 5' end of *cyaA* was amplified by PCR with oligonucleotides (MWG Biotech, Germany) PAB5 5'-CGCCGGTACCATGCAGCAATCGCATCAGGCT-3' and PAB6 5'-TGGTGAATTCGCTCTTGCCCG-3'. The resulting product was digested with *Kpn*I and *Eco*RI (Invitrogen, CA, USA), inserted in corresponding sites of the cloning vector pBluescript SK- (Stratagene, CA, USA), and this plasmid was named pAPB4. The 3' end of *cyaA* was amplified by PCR from *B. pertussis* genomic DNA with oligonucleotides PAB7 5'-AAGAGCGAATTCACCACATTCGTCG-3' and PAB2 5'-CGCGGATCCTCAGCGCCAGTTGACAGCCA-3'. The product was digested with *Eco*RI and *Bam*HI and ligated into pBluescript SK- at the same restriction sites. This plasmid was named pAPB5, and was then digested with *Eco*RI and *Bam*HI and the 3' *cyaA* fragment was subcloned into the corresponding sites of pAPB4 giving a full-length *cyaA* gene. This plasmid was named pAPB6. *cyaC* was amplified by PCR from the genomic DNA of *B. pertussis* with oligonucleotides PAB3 5'-CGCGGATCCGAGGGCATGTCATGCTTCCGTCCGCC-3' and PAB4 5'-CGCGGCGAAGCTTTCAGGCGGTGCCCCGGC-3'. The PCR fragment was

digested with *Bam*HI and *Hin*DIII and cloned into the pASK-IAB6 (IBA GmbH, Germany) expression vector opened with the same restriction enzymes. This new plasmid was termed pAPB1. The intact *cyaA* gene was isolated from pAPB6 digested with *Kpn*I and *Bam*HI, cloned into pAPB1 upstream of the *cyaC* gene using the *Kpn*I and *Bam*HI sites, and this plasmid was termed pAPB8. pAPB8 was digested with *Kpn*I and *Hin*DIII and the 5.9 kb product containing *cyaA* and *cyaC* was cloned into the commercial His-tagged vector pQE-80 (Qiagen, UK) opened at the same restriction sites. The sequence and orientation of cloned genes were confirmed by restriction digestion and sequencing (MWG Biotech). This plasmid was named pJR2 (SEQ ID No. 8) from which His-tagged palmitoylated CyaA could be expressed in *E. coli*.

Construction of Plasmids Expression CyaA and CyaA derivatives. Plasmid pJR1 (SEQ ID No. 5) was constructed in the following manner. *cyaA* was subcloned as a *KpnI/BamHI* fragment from pAPB6 (pBluescript SK- containing *cyaA*) into pASK-IBA6 (IBA) to generate plasmid pAPB7. A *KpnI/Hin*DIII fragment comprising *cyaA* from pAPB7 was then cloned into the corresponding sites of pQE-80 (Qiagen) to generate pJR1. Plasmid pAPB9 was constructed by inserting a 3.5 kb *Sst*I fragment encoding the 5' end of *cyaA* into the corresponding site in pBluescript SK-. Using pAPB9 as a template, a 0.4 kb PCR product was generated with oligonucleotides pUC Forward (CCCAGTCACGACGTTGTAAAACG) - (Stratagene, CA, USA), and PAB27
(CAACCCCCAATCGGA*TCCCGC*GGCGG*GC*CACGCCCAATCCTTTG – introduced *Bam*HI site underlined, mutated bases in italics) and a 1.7 kb product with oligonucleotides PAB17
(CAAAGGATTGGGCGTG*GC*CGCC*GC*G*GGATCC*GATTGGGGGTTG – introduced *Bam*HI site underlined) and PAB29
(CGTAGATCTCCATGGGACTGAGC – *Nco*I site underlined). The former PCR product was digested with *Xba*I/*Bam*HI and the latter with *Bam*HI/*Nco*I and ligated with *Xba*I/*Nco*I-digested pAPB9 to create pNM1. The 2.5 kb *Cla*I/*Kpn*I fragment of pNM1 was inserted into the corresponding sites of pJR1 and pJR2 to create pAPB22

(SEQ ID No. 7) and pNM2 (SEQ ID No. 1), respectively. These plasmids encode a H63A/K65A/S66G mutant of His-CyaA, either alone (pAPB22) or together with CyaC (pNM2). The sequence and orientation of the cloned genes were confirmed by restriction digestion and sequencing (MWG Biotech).

Purification of CyaA. *E. coli* XL-1 Blue (pJR2) was induced to express CyaA and CyaC by the addition of isopropyl-β-thiogalactopyranoside (IPTG, Bioline, UK) to an exponentially growing bacterial culture in Luria-Bertani (LB) broth supplemented with 150 μg/ml ampicillin with vigorous shaking at 37^0C. The bacterial culture was centrifuged and the bacterial pellet resuspended in 50 mM Tris-HCl, 0.2 mM $CaCl_2$, pH 8.0, supplemented with protease inhibitor cocktail (P-8465 Sigma, UK). Bacteria were disrupted with FastPrep Protein Blue beads (QbioGene, CA, USA) in a FastPrep machine at speed 6 for 20 sec. The insoluble material containing CyaA was separated by centrifugation, washed with 50 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2% Triton X-100, pH 8.0 and incubated in 50 mM Tris-HCl, 0.2 mM $CaCl_2$, 8 M urea, pH 8.0 (Buffer A) for 1 h at room temperature with stirring. The solubilised CyaA was collected following centrifugation. After addition of NaCl to a final concentration of 0.1 M, CyaA was loaded on a DEAE cellulose (Sigma) column equilibrated with Buffer A supplemented with 0.1 M NaCl and eluted with Buffer A supplemented with 0.2 M NaCl. The protein was further purified on Ni^{++} columns (Qiagen) under denaturing conditions by pH adjustment as recommended by the manufacturers and eluted in 100 mM $NaHPO_4$, 10 mM Tris-HCl, 8 M urea, 0.2 mM $CaCl_2$, pH 4.5. LPS removal was attempted using Detoxigel endotoxin removal columns (polymyxin B conjugated columns, Pierce, IL, USA) following the manufacturer's protocols. LPS was dissociated from CyaA by dialysis first against Dulbecco's PBS (Sigma), 1 mM EDTA, 1 M urea, pH 4.6 and then against Dulbecco's PBS, 0.1 mM $CaCl_2$, 2 M urea pH 8.0. The purified protein was stored in aliquots at -20 C. LPS was measured by a colourimetric limulus amaeobocyte lysate assay (QCL-1000; Biowhittaker, MD, USA) and protein concentrations were determined by Bradford (Bio-Rad). Proteins were separated by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualised with

Coomassie Blue (GelCode Blue Stain Reagent, Pierce). Alternatively proteins were transferred to a nitrocellulose membrane following SDS-PAGE and probed with anti-His Tag antibodies (Santa Cruz Biotechnologies) and anti-CyaA antibodies (kind gift from Erik Hewlett). The bands were visualised by incubation with secondary anti-rabbit IgG horseradish peroxidase conjugated antibodies (Sigma) and chemiluminescent supersignal detection system (Pierce).

Purification of CyaA and CyaA derivatives. The CyaA proteins were expressed and purified from *E. coli* XL-1 Blue carrying either plasmid pJR1 (expressing His-CyaA alone (SEQ ID No.4)), pJR2 (expressing His-CyaA and CyaC together (SEQ ID No. 8)), pNM2 (expressing enzymatically inactive His-CyaA and CyaC (SEQ ID No. 1)) or pAPB22 (expressing enzymatically inactive His-CyaA (SEQ ID No. 7)). The N-terminal His-tagged proteins were purified from inclusion bodies by diethylaminoethyl–sepharose and Ni^{++}-agarose chromatography and contaminating LPS was removed by dialysis against EDTA-containing buffers of low pH. All chemicals were from Sigma, unless indicated otherwise. LPS was measured by a highly sensitive colourimetric limulus amaeobocyte lysate assay (Cape Cod Associates) and protein concentrations were determined by Bradford assay (Bio-Rad). Proteins were separated by SDS-PAGE and visualised with Coomassie Blue. The proteins were judged to be greater than 95% pure by Coomassie stained SDS-PAGE gels. Alternatively, proteins were transferred to nitrocellulose membrane following SDS-PAGE and probed with anti-His antibodies (Santa Cruz Biotechnologies) and anti-CyaA antibodies (kind gift from Erik Hewlett). The bands were visualised by incubation with secondary anti-rabbit IgG horseradish peroxidase conjugated antibodies and chemiluminescence detection system (Pierce). Each protein was recognised by both antibodies.

Palmitoylation of CyaA. The co-expression of *cyaC* with *cyaA* in *E. coli* produces a CyaA protein that is acylated with palmitoyl fatty acids at Lys^{983} and Lys^{860}. Expression of *cyaA* alone in the same system results in a CyaA protein devoid of this post-translational modification. To confirm the acylation status of our preparations of

NA-CyaA and A-CyaA, we used a radiolabeling technique. *E. coli* (strain XL1-blue) harbouring the plasmids pJR1 (encoding *cyaA*) and pJR2 (encoding *cyaA* and *cyaC*) were streaked from glycerol stocks and grown on LB plates with ampicillin (150 μg/ml) o/n at 37°C. Single colonies were inoculated in a liquid 5 ml culture of LB+Amp and grown o/n with shaking at 37°C. This was then diluted to a 5 ml culture of OD600=0.2 and was grown until OD=0.4-0.6. The bacteria from 1 ml of this culture were harvested by centrifugation at 800g for 10 min in a desktop microfuge and resuspended in 1ml LB broth with ampicillin (150 μg/ml), supplemented with 5μCi of [^{14}C(U)] palmitic acid (0.1 mCi/ml; Perkin Elmer, MA USA) by shaking vigorously at room temperature for 1 h. Protein expression was induced with 1 mM IPTG for 15 min. The cells were harvested and washed in fresh medium, pelleted and re-suspended in SDS-PAGE loading dye and the equivalent of 300 μl of bacterial culture was electrophoresed on a 7% SDS-PAGE gel, which was then stained with gel-code blue reagent (Pierce) to visualise the proteins and identify the CyaA protein band. Highly purified recombinant A-CyaA (not radioactively labelled; 2 μg) was also run on the same gel to identify the recombinant protein in the bacterial lysate. Fluorography was then used to visualise the radio-labelled palmitoylated proteins on the gels.

In vitro CyaA enzymatic activity. 0.1 μg protein was incubated for 5 min at 30^{0}C in 50 μl 50 mM Tris-HCl, 100 μg/ml BSA, 0.1 μM calmodulin, 0.12 mM $CaCl_2$, 6 mM $MgCl_2$, 2 mM ATP, pH 8.0. The reaction was mixed with lysis reagent 1B of the Amersham Biosciences Biotrak enzymeimmunoassay system and boiled for 5 min. cAMP was measured by a competitive ELISA in the Amersham kit. Units are μmol cAMP produced per min at 30ºC, pH 8.0.

cAMP Quantification. To assay intracellular cAMP accumulation, CyaA or CyaA-derivatives were added at a concentration of 0.1-10 g/ml to J774 macrophages or dendritic cells. cAMP was measured by competitive ELISA using the Amersham Biosciences Biotrak Enzymeimmunoassay kit. Samples were serially dilutes to obtain values within the linear range of the concentration curve.

Lactic dehydrogenase (LDH) Assay. The lysis of J774 macrophages was measured by the release of LDH into the culture supernatants. Toxin was added to the cells at the indicated concentrations and the plates incubated at 37 C for 6 h. LDH activity in the supernatants was quantified by the CytoTox 96 assay (Promega). Percentage lysis = [(OD of sample - OD of untreated cells)/(OD of 100% lysis cells - OD of untreated cells)]*100.

Morphological assessment of CyaA treated J774 cells. J774 cells were plated on glass coverslips one day before treatment. Cells were treated with 1-10 μg/ml of CyaA or NA-CyaA, 1 μM cyclohexamide or medium only for 6 h. After treatment, coverslips were removed and cells were fixed with 3% paraformaldehyde in PBS, washed and mounted on glass slides for analysis. Cells were visualised under white light using a BX51 microscope (Olympus) with attached camera and images acquired using AnalySIS® software.

Caspase-3 Activation Assay. J774 cells were treated for 6 h with CyaA or CyaA derivatives (0.1 – 10 μg/ml) in cDMEM lacking foetal calf serum. Cells were washed and lysed in 300 μl lysis buffer (150 mM NaCl, 20 mM Tris (pH 7.5), 1% Triton X-100, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml aprotinin) and analyzed for protein concentration. 50 l s amples were added to 50 l of 2X reaction buffer (100 mM HEPES (pH 7.4), 150 mM NaC, I0.2% CHAPS, 4mM DTT) and 50μM caspase-3 substrate (Ac-DEVD-AFC, Alexis Corp.) and incubated at 37°C. Fluorescence caused by liberation of free AFC was monitored over 60 min (excitation, 390 nm; emission, 510 nm).

Macrophages and DC. The J774 murine macrophage cell line was cultured in complete DMEM (cDMEM; DMEM supplemented with 8% foetal calf serum, 100 mM L-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin) and passaged every 3-4 days. Bone marrow–derived immature DC were prepared by culturing bone marrow cells obtained from the femur and tibia of mice in complete RPMI-1640

(cRPMI; RPMI supplemented with 8% foetal calf serum, 100 mM L-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin) supplemented with supernatant (10%) from a granulocyte-macrophage-colony stimulating factor (GM-CSF) expressing cell line (J558-GM-CSF). On day 3, fresh medium with 10% GM-CSF cell supernatant was added to adherent cells. On day 7, cells were collected, washed, recultured in cRPMI and used for assays. J774 cells and DC were used in experiments at a concentration of $1x10^6$ cells/ml.

Effects of CyaA on Cytokine Release by Macrophages and DC. CyaA was added at the indicated concentrations to macrophages and DC. Where indicated, purified anti-mouse CD11b antibody (M1/70) or purified rat IgG_{2b}, isotype control (both from PharMingen) were added at a concentration of 10 g/m l, 30 min prior to the addition of CyaA. Polymyxin B was added to the appropriate wells at a concentration of 10 g/ml. 2 h after stimulation with CyaA, LPS or phosphoro-thioate-stabilised oligodeoxynucleotide-containing CpG motifs (CpG-ODN; 5'-GCTAGACGTTAGCGT-3'; synthesized by Sigma-Genosys Ltd) was added at the indicated concentrations. Supernatants were collected after 2, 4 24 or 28 h for analysis of IL-10, IL-12 p70, TNF- , IL-6 and CCL3 concentrations by ELISA (R&D DuoSet ELISA kits).

Effects of CyaA on DC maturation—DC were cultured with CyaA (1 g/ml) and polymyxin B (10 g/ml). 2 h later 10 ng/ml or 1μg/ml LPS was added. After 24 h incubation cells were collected, washed in PBS with 0.05% bovine serum albumin and 0.02% NaN_3. Cells were incubated for 30 min at 4°C with antibodies specific for mouse CD80 (Hamster IgG2, clone 16-10A1), CD86 (Rat IgG2a, clone GL1), CD11c (Hamster IgG1, clone HL3), MHC class II (mouse IgG2b, $I\text{-}A^d$, clone AMS-32.1), CD40 (Rat IgG2a, clone 3/23) or ICAM-I (Hamster IgG1, clone 3E2), followed by washing and incubation with streptavidin-PerCP in the case of biotin labelled primary antibodies. Cells labelled with appropriate isotype matched antibodies with irrelevant specificity acted as controls. 30,000 cells per sample were analysed on a FACScaliber flow cytometer. Analysis was performed on CD11c

gated cells using CellQuest V3.3 software (Becton Dickinson Immunocytmetery Systems, San Jose, CA).

Animals and immunization. Female specific pathogen free BALB/c, C3H/HeN and C3H/HeJ mice were purchased from Harlan Olac (Bicester, UK) and used at 6-8 wk old, with 4 or 5 mice per group. Mice were housed in individually ventilated cages and all experiments were performed according to regulations of the Irish Department of Health, the EU and the Ethics Committee of Trinity College Dublin.

Mice were immunized subcutaneously (s.c.) in the hind footpads once or twice (0 and 21 days) with depyrogenated keyhole limpet hemocyanin (KLH; 5 μg; Calbiochem, La Jolla, CA, USA), KLH (5 μg) with CyaA or NA-CyaA (1 μg) or with Dulbeccos PBS (Sigma, Poole, UK) in a final volume of 50 μl. Seven days after the first or second immunization mice were sacrificed by cervical dislocation and serum and popliteal lymph nodes collected.

Generation of Ag-specific T cell lines and clones. Popliteal lymph node cells (1 x 10^6/ml) from immunized mice were cultured with KLH (50 μg/ml). After two round of antigen stimulation, T cell lines were cloned by limiting dilution as described (37). T cell lines and clones were maintained by culture with antigen (KLH, 50 μg/ml) and splenic APC for 4-5 days, followed by 5-7 days culture with irradiated feeder cells and IL-2. T cells were tested for cytokine production at the end of the starve cycle.

Antigen-specific cytokine production. Lymph node cells (1 x 10^6 cells/ml) from immunized mice or T cell lines or clones and APC (irradiated spleen cells, 2 x 10^6/ml) were cultured at 37ºC and 5% CO_2 in RPMI medium with KLH (2-50 μg/ml) or phorbol 12-myristate 13-acetate (PMA; 25 ng/ml; Sigma) and anti-CD3 (0.5μg/ml; BD, Pharmingen) or medium only. After three days, supernatants were collected for cytokine detection and the medium replaced. On the following day ^{3}H-Thymidine (950 μCi/well; Amersham Pharmacia, UK) was added and the cells cultured for a further 5 h, after which cells were harvested and proliferation assessed

by ^{3}H-Thymadine incorporation. Concentrations of IL-4, IL-5 and IFN-γ were determined by immunoassay using pairs of antibodies and recombinant cytokines (BD Pharmingen, San Diego, CA) as standards. IL-10 concentrations were determined using a commercially available Duo-Set kit kits (R&D Systems, Minneapolis, U.S.A.)

Antibody assays. Titres of KLH-specific IgG, IgG1 and IgG2a in the serum of immunized mice were determined by ELISA.

Statistics. Cytokine and Chemokine levels were compared by one-way analysis of variance (ANOVA). Where significant differences were found, the Tukey Kramer multiple comparisons test was used to identify differences between individual groups.

Cloning, expression and purification of CyaA. *cyaA*, the gene encoding CyaA, and *cyaC*, the gene whose product is required to post-translationally activate CyaA, were cloned from the genomic DNA of *B. pertussis* W28 into pQE-80 to allow inducible expression of these genes in *E. coli*. This plasmid pJR2, expressing 6xHis-tagged CyaA, was introduced into electrocompetent *E. coli* XL1-blue cells. Bacteria harbouring the recombinant plasmid were recovered and the correct orientation and position of the cloned genes were confirmed by both restriction digestion and sequencing.

Active CyaA was solubilised from bacterial inclusion bodies and purified on DEAE and Ni^{++} columns. The purity of the 200 kDa CyaA protein was greater than 95% as estimated by Coomassie staining following gel electrophoresis and this band was recognised by both anti-His and anti-CyaA antibodies (data not shown). The LPS content of the CyaA protein was monitored throughout the purification process. At this stage the protein preparation contained significant amounts of LPS (>2 ng LPS/μg protein). A number of procedures were used to attempt to remove the LPS. Passage of the protein preparation through polymyxin B conjugated columns

reduced, but did not eliminate, the LPS. This suggested that CyaA was closely complexed with LPS. LPS can be dissociated from proteins by the addition of EDTA to chelate calcium ions and by lowering the pH to below the pI of the protein. After dialysis of the protein in these conditions, the preparation of CyaA contained 220 pg LPS /μg protein. This concentration of LPS did not stimulate macrophages or DC *in vitro* (data not shown; and also Fig. 4, 5 and 7). This preparation was used in the adjuvant and immunomodulatory studies described below.

CyaA biochemical properties. The CyaA preparation was analysed biochemically to ensure that both its enzymatic and membrane translocation properties were active. *In vitro* assays of the adenylate cyclase enzyme function showed that the protein was enzymatically active (26.6 ± 0.8 units/mg). CyaA at a concentration of 1 μg/ml was able to increase the intracellular concentration of cAMP 80-fold in J774 macrophages (72.8 ± 2.9 vs 0.9 ± 0.1 pmol cAMP/10^6 cells), showing that the protein has the ability to target and enter eukaryotic cells. The maximal cAMP concentration was reached within 30 min and was maintained for at least 24 h. This concentration of CyaA induced less than 1% cell lysis after 24 h, as measured by LDH release, however lysis (up to 10%) was observed with CyaA at concentrations of 5–10 μg/ml (data not shown). Therefore to assess its immunomodulatory function CyaA was used at 1 μg/ml, a concentration that induces a large increase in intracellular cAMP, without affecting cell viability.

CyaA generates Th2 and Tr1 cells to co-injected antigen. To examine the adjuvant properties of CyaA, mice were immunized s.c. in the hind footpad with KLH (5 μg), alone or with CyaA (1 μg). Seven days later mice were sacrificed and lymph node cells were re-stimulated with antigen (KLH 2-50 μg/ml) *in vitro.* Cytokine concentrations were determined in supernatants removed after 3 days and proliferation was assessed after 4 days. Immunization with KLH alone induced weak cellular immune response; only IL-4 production was enhanced over that observed in mice immunized with PBS (Fig. 1). In contrast, significant antigen-specific proliferation was observed in cells from the re-stimulated lymph node cells from

mice immunized with KLH and CyaA. Furthermore, significantly higher concentrations of KLH-specific IL-10 and IL-5 were detected in lymph node cells from mice immunized with CyaA and KLH compared to mice immunized with KLH alone. IL-4 and IFN-γ were also enhanced, but the difference between mice that received KLH alone and KLH and CyaA was, in most cases, not significant. A similar pattern of cytokine secretion was observed 7 days after a booster immunization (data not shown).

The cytokine profile of antigen-stimulated lymph node cells suggested that CyaA enhances Th2 and /or Tr1 cells to co-administered antigens. In order to confirm this finding, we generated KLH-specific $CD4^+$ T cell lines and clones from mice immunized with KLH in the presence of CyaA. Each of the 10 T cell lines examined secreted high levels of IL-10 and lower levels of IL-5 and a smaller number also secreted IL-4 (Fig. 2A). IFN-γ production was detectable in 6 of 10 T cell lines examined and at high levels in only one of these T cell lines (Fig. 2A). In contrast, IFN-γ was produced at concentration in excess of 50 ng/ml by all T cell lines from mice immunized with KLH in the presence of CpG oligodeoxynucleotides (unpublished observations). A number of the T cell lines generated from mice immunized with KLH and CyaA were cloned and cytokine-production by T cell clones from one representative T cell line is shown in Fig. 2B. These KLH-specific T cell clones secreted IL-5 and IL-10, or IL-4, IL-5 and IL-10 but undetectable IFN-γ, profiles characteristic of Tr1 and Th2 cells respectively. These findings demonstrate that CyaA promotes the induction of Th2 and Tr1-type cells specific for the co-administered antigen.

CyaA enhances IgG1 responses to co-administered antigen. We examined the adjuvant effect of CyaA for antibody responses to co-injected antigen by assessing KLH-specific IgG and IgG subclasses in mice immunized with KLH alone or with CyaA. Significantly higher levels of KLH-specific IgG1 were found in the serum of mice immunized with KLH and CyaA compared with mice that received antigen alone. In contrast, CyaA did not enhance IgG2a levels above those observed in mice

immunized with KLH alone (Fig. 3A). Following a second immunization the serum IgG titres were increased over those observed after a single immunization and the responses to KLH in the presence of CyaA were significantly greater than those in mice immunized with KLH alone (Fig. 3B). Comparable with the data after a single immunization, IgG1 was the dominant subclass of the antibody response. These data clearly demonstrate that CyaA acts an adjuvant for antibody, as well as T cell responses *in vivo*.

CyaA modulates cytokine production from innate cells. Cells of the innate immune system, including DC and macrophages direct the adaptive immune response by presenting antigens and secreting regulatory cytokines. To investigate the effect of CyaA on these cells, J774 macrophages and immature bone marrow-derived DC were incubated with CyaA (1 μg/ml), LPS (1-1000 ng/ml) or CyaA and LPS. Since the CyaA protein is associated with LPS, which was reduced but not completely eliminated during purification, it was important to determine the role, if any, of this LPS in the immunomodulatory effects of CyaA. Therefore cells were also stimulated with CyaA in the presence of polymyxin B. Supernatants were collected 2, 4 and 28 h after stimulation and assayed for cytokines. The purified CyaA, that included residual LPS (220 pg/ml), stimulated low levels of IL-6, IL-10 and TNF-α production from J774 cells (Fig. 4) and low levels of IL-6 and TNF-α (but no IL-10) secretion from DC (Fig. 5). This cytokine production was abrogated in the presence of polymyxin B. However, stimulation with LPS alone at the dose present in the CyaA preparation (220 pg/ml) did not induce production of these cytokines (Figs 4 and 5). These data suggest that CyaA activates innate cells only in the presence of LPS. Therefore we examined the effect of CyaA on cytokine production in response to increasing doses of LPS. CyaA synergised with LPS in promoting IL-6 and IL-10 production from macrophages and DC. IL-10 production from macrophages stimulated with CyaA and LPS (1-1000ng/ml) was significantly higher than that of macrophages stimulated with the corresponding dose of LPS alone at all time points examined (Fig. 4). IL-10 could not be detected in DC supernatants 4 h after stimulation with LPS (1-1000 ng/ml) alone, whereas

significant levels of IL-10 were produced following addition of CyaA (Fig. 5). LPS-induced IL-6 production by macrophages and DC was also significantly enhanced by the addition of CyaA, but this was only observed at early time points. In contrast to the positive effect on IL-6 and IL-10 production, CyaA suppressed TNF-α secretion from macrophages and DC and IL-12p70 production from DC. These inhibitory effects were observed at the three time points examined and over a range of doses of LPS. These data demonstrate that CyaA alone has little enhancing effect on cytokine production by cells of the innate immune system, but can synergise with LPS, even at very low concentrations, in promoting IL-6 and IL-10 production, but also inhibiting TNF-α and IL-12 production.

Effect of CyaA on maturation of DC. Several pathogen-derived molecules that bind to TLRs, induce maturation of immature DC, thereby enhancing their capacity to activate naïve T cells. Therefore, we examined the ability of CyaA to stimulate the maturation of DC and/or to modulate LPS-induced maturation. Immature DC were stimulated with CyaA, LPS or LPS with CyaA and the expression of surface markers associated with maturation was examined by immunoflourescence analysis 24 h later. As expected, LPS (1 μg/ml), enhanced surface expression of CD80, CD86, ICAM-I, CD40, and MHC class II (Fig. 6). Stimulation of immature DC with CyaA (in the presence of polymyxin B) also resulted in upregulation of surface expression of CD80 and MHC class II, and to a lesser extent CD86. In contrast, expression of CD40 and I-CAM I was downregulated following incubation with CyaA. Furthermore, CyaA inhibited LPS-induced upregulation of CD40, ICAM-I and CD86. In contrast, treatment of cells with LPS at the level present in the CyaA preparation (220 pg/ml) had no effect on DC surface marker expression (data not shown). These findings demonstrate that CyaA treatment results in partial maturation of the DC, upregulating CD80 and MHC-II, but inhibits CD40 and ICAM-1, a phenotype drives the differentiation of Tr1 cells.

Effect of CyaA on DC from TLR-4 defective mice. To further address the role of LPS in the mechanism of action of CyaA as an adjuvant and immunomodulator, we

examined innate cytokine and chemokine production and maturation of DC from TLR4-defective mice. DC from C3H/HeN and C3H/HeJ mice were treated with CyaA, LPS, LPS and CyaA or CyaA and polymyxin B and supernatants recovered after 4 h. CyaA (that included 220 pg/ ml residual LPS) stimulated IL-10 production by DC from C3H/HeN, but not from C3H/HeJ mice (Fig. 7). Furthermore, IL-10 production by DC from C3H/HeN mice was inhibited by polymyxin B. However, LPS alone at the concentration present in the CyaA preparation (220 pg/μg) did not induce IL-10, IL-12p70, TNF-α or MIP-1α production by DC from C3H/HeN mice. Addition of a higher dose of exogenous LPS (10 ng/ml) did not induce IL-10 at the 4 h time point, but synergized with CyaA in promoting IL-10 production. Furthermore, CyaA suppressed LPS induced IL-12p70, TNF-α and MIP1-α production by DC from C3H/HeN mice (Fig. 7), but had no effect on cytokine production by DC from C3H/HeJ mice (Fig. 7). In contrast, CpG, a TLR9 ligand, activated cytokine production by C3H/HeN and C3H/HeJ DC in a similar fashion (Fig. 7). These data provide further evidence that CyaA, although having no direct effect on cytokine production by DC, modulates LPS-induced responses, synergising with LPS to induce IL-10 and suppressing LPS induced production of IL-12p70, TNF-α and MIP-1α.

We also examined the effects of CyaA on maturation of DC from C3H/HeN and C3H/HeJ mice. As shown for BALB/c mice, CyaA induced maturation of DC from C3H/HeN mice, specifically CyaA enhanced expression of CD80, CD86, MHC class II, CD40 and ICAM-1 (Fig 8A). In the presence of polymyxin B these effects were diminished, in particular CD40, ICAM-1, and MHC class II, which were expressed at lower levels than those seen on medium-treated control DC. LPS-induced expression of CD86, CD40 and ICAM-1 was also inhibited by CyaA, though not to the same extent as that observed in DC from BALB/c mice. In contrast to the modulatory effects in C3H/HeN mice, LPS, CyaA or LPS with CyaA had no enhancing effects on CD86, MHC class II, CD40 or ICAM-1 and had a modest effect on CD80 on DC from C3H/HeJ mice (Fig. 8B). However CyaA marginally reduced CD40 and ICAM-1 expression on DC from C3H/HeJ mice in the presence

of polymyxin B. These findings suggest that upregulation of maturation markers on DC by CyaA is dependent on LPS even at very low doses, but that inhibition of endogenous expression of CD40 and ICAM-1 can occur in the absence of LPS.

Purification and biochemical characterisation of acylated, non-acylated and enzymatically inactive CyaA. The following studies examined the role of acylation and of enzyme activity on the ability of CyaA to modulate immune responses. Acylation is required for CyaA to interact with red blood cells and the adenylate cyclase activity is responsible for the accumulation of cAMP in host cells. In order to examine the role of acylation and adenylate cyclase activity in the adjuvant and immunomodulatory effects of CyaA, we generated and purified CyaA and CyaA derivatives lacking acylation and/or deficient in adenylate cyclase activity. Recombinant N-terminal His-tagged fusion proteins of CyaA and CyaA derivatives were expressed and purified from *E. coli*. Acylated CyaA (A-CyaA) was purified from *E. coli* XL-1 Blue (pJR2) expressing His-CyaA and CyaC together under the control of the IPTG-inducible promoter p_{tac}. Non-acylated CyaA (NA-CyaA) was purified from *E. coli* carrying pJR1, a similar plasmid that lacks *cyaC*. CyaA protein with an inactive adenylate cyclase domain (iAC-CyaA) was generated by site directed mutagenesis of the *cyaA* gene in a region of the gene encoding amino acids known to be involved in the catalytic activity of this protein. The mutated protein had H63A, K65A and S66G substitutions - Lys^{65} is important for binding ATP and His^{63} is involved in its cyclisation (21, 22). Acylated iAC-CyaA (A-iAC-CyaA' SEQ and non-acylated iAC-CyaA (NA-iAC-CyaA) (SEQ ID No. 3) were expressed in and purified from *E. coli* in the presence or absence of CyaC, respectively. LPS appears to be complexed with CyaA, and the LPS content of purified preparations used in this study ranged from 124 to 217 pg LPS/ g CyaA. The DNA sequences of wildtype *cyaA* and *cyaA*-derivatives were confirmed by sequencing of the cloned genes.

The presence and absence of palmitic fatty acid acylation on A-CyaA and NA-CyaA respectively, was confirmed by inducing protein expression in *E. coli* grown in the

presence of [^{14}C(U)] palmitic acid. The results shown in Fig. 9 demonstrate that the A-CyaA preparation is modified by post-translational palmitoylation by the co-expressed accessory protein CyaC, whereas, CyaA expressed in the absence of CyaC (NA-CyaA) does not become palmitoylated.

Both A-CyaA and NA-CyaA were enzymatically active, with specific activities of 47 and 31 mol cAMP generated/min/mg respectively for preparation 1 and specific activities of 105 and 121 mol cAMP generated/min/mg respectively for preparation 2, while neither acylated nor non-acylated iAC-CyaA proteins exhibited enzyme activity (Fig. 11A).

Acylation of CyaA is required for haemolysis of red blood cells and lysis and caspase-3 activation in macrophages. Acylation had previously been shown to be necessary for CyaA to bind to, increase intracellular cAMP in and cause haemolysis of red blood cells (1). Furthermore, non-acylated CyaA was unable to lyse J774 macrophages or Jurkat T-cells, but the ability of NA-CyaA to bind to or cause cAMP accumulation in these cells was not reported (1, 2). Recently it was reported that NA-CyaA was unable to lyse or to increase cAMP concentrations in CHO cells transfected with CD11b, however, it could efficiently bind these cells in a CD11b-dependant manner, but with lower affinity than A-CyaA (14) We examined the role of acylation and enzymatic activity in CyaA-induced cAMP accumulation in J774 macrophages, which express CD11b/CD18. The enzymatically active A-CyaA and NA-CyaA increased the intracellular cAMP concentration in J774 cells by approximately 1000–fold, but A-iAC-CyaA and NA-iAC-CyaA did not alter cAMP concentration (Fig. 11B). The increase in intracellular cAMP was lower with NA-CyaA than A-CyaA. Nevertheless, our results clearly demonstrate that acylation is not essential for induction of intracellular cAMP as NA-CyaA enhanced intracellular cAMP over a wide concentration range (Fig. 12).

CyaA induced haemolysis in red blood cells, whereas NA-CyaA had no effect (Fig 10). We also assessed the ability of these proteins to cause lysis of macrophages. At

a concentration of 10 g/ml, both of the acylated CyaA proteins induced cell death in J774 macrophages, however only minimal lysis was detected with NA-CyaA or NA-iAC-CyaA (Fig 11C). Following incubation with polymyxin B, the low level of lysis detected with 10 μg/ml of NA-CyaA was abrogated and the lysis induced with A-CyaA was reduced (compare Fig. 12C and Fig. 12D). Thus, acylation is required for CyaA to lyse cells, as is the case for other RTX toxins, moreover, LPS can augment this lytic effect. In contrast, enzymatic activity and enhancement of intercellular cAMP concentration are only minimally associated with the ability of CyaA to cause cell lysis.

The difference in cytotoxicity between A-CyaA and NA-CyaA was confirmed by examination of the morphology of CyaA-treated macrophages. The morphology of NA-CyaA treated cells was similar to that of untreated cells (Fig. 13). In contrast, J774 cells treated with A-CyaA at concentrations of 3 or 5 μg/ml (Fig. 13 B and C) take on apoptotic morphology, with cytoplasm and nuclear shrinkage as well as cellular fragmentation (nuclear staining of cells with Hoechst stain showed clear chromatin condensation; data not shown). This compares with the obvious apoptotic morphology induced by cycloheximide (Fig. 13E). At higher concentrations of CyaA (Fig. 13 D) a phenotype more characteristic of necrosis is apparent, with cytoplasmic and nuclear swelling visible and obvious vacuolisation of the cytoplasm. A key step in apoptosis is the activation of the protease, caspase-3. Therefore we examined caspase-3 activation in cells treated with A-CyaA and NA-CyaA. A-CyaA at concentrations of 5 and 10 μg/ml induced high levels of caspase-3 activity and 3 μg/ml induced lower levels of activation (Fig 11D and 3B). Similarly, A-iAC-CyaA induced caspase-3 activation (Fig. 11D), whereas NA-CyaA did not induce significant caspase-3 activation at concentrations in the range 0.3-10 μg/ml (Fig. 11D and B). Caspase-3 activation was only detected with the highest concentration of NA-iAC-CyaA tested (10 μg/ml). These results indicate that acylation facilitates CyaA-induced apoptosis, and that the absence of enzymatic activity may in part compensate for lack of acylation, possibly by removal of an inhibitory effect of cAMP on caspase-3 activation.

Acylation is not required for the cAMP driven modulation of cytokine release by CyaA-treated macrophages. We examined the influence of acylation and enzymatic activity on the ability of CyaA to modulate macrophage and DC activation. J774 macrophages were treated with either CyaA alone (1 μg/ml), CyaA together with LPS (10 ng/ml) or CyaA and polymyxin B (10 μg/ml) to negate any effects of the low concentrations of remaining LPS in the protein preparations. The concentrations of secreted IL-10 and TNF- were quantified in supernatants 4h later. NA-CyaA synergised with LPS to induce IL-10 secretion in a similar fashion to A-CyaA (Fig. 5). A-CyaA and NA-CyaA proteins (with or without added LPS) induced IL-10 production by macrophages. Addition of polymyxin B, which chelates LPS, abrogated the production of IL-10. LPS alone, at the concentrations present in the CyaA protein preparations, failed to induce IL-10 production, indicating that this cytokine was secreted as a result of the synergy between LPS and CyaA in each of the protein preparations. This synergy was evident with LPS at the concentrations present in the protein preparation (approximately 200 pg/ml LPS in these samples) and was not significantly altered by further addition of exogenous LPS (10 ng/ml). NA-CyaA and A-CyaA inhibited LPS-induced TNF- secretion to the same extent. In contrast, neither of the iAC-CyaA proteins enhanced IL-10 production or inhibited TNF- production (Fig. 14), indicating that adenylate cyclase activity is essential for CyaA to modulate cytokine production by macrophages. The enzymatically inactive toxin preparations (NA-iAC-CyaA and NA-iAC-CyaA) induce TNF-α production in the absence of additional exogenous LPS. This is due to the LPS, which still remains complexed with the proteins after extensive purification, as polymyxin B reduces this TNF-α to baseline concentrations. This effect is not seen with the enzymatically active toxins, because of the inhibitory activity of cAMP on TNF-α production.

In order to further investigate the role of acylation in the immunomodulatory activity of CyaA, we examined the effect of the proteins over a range of concentrations (0.1 to 3 g/ml) on LPS-induced cytokine production (Fig. 15). At concentrations of 1 g/ml and above NA-CyaA and A-CyaA exert similar effects, enhancing IL-10 and inhibiting TNF-α production by macrophages, while at lower concentrations NA-

CyaA had significantly less modulatory activity than A-CyaA. These data indicate that acylation is not an absolute requirement for CyaA to modulate macrophage cytokine release, but it does increase its efficiency. Likewise acylation did not influence the ability of CyaA to modulate cytokine and chemokine production by bone marrow-derived DC from BALB/c or C3H/HeN mice (data not shown; see also Fig. 16).

NA-CyaA modulates the effects of CpG-ODN signalling through Toll-like receptor (TLR)-9. In order to determine whether the synergistic and inhibitory effects of CyaA for IL-10 and proinflammatory cytokine production are limited to LPS, which is closely associated with the protein, or extend to other TLR ligands, we examined the effect of CyaA on cytokine production induced by a TLR-9 ligand, CpG-ODN. To eliminate any possible influence of LPS, these studies were performed with DC generated from C3H/HeJ mice, which have a point mutation in TLR-4 that renders them hyporesponsive to LPS. CpG-ODN-stimulated TNF-, IL-12p70 and CCL3 production by C3H/HeJ DC was downregulated by A-CyaA and NA-CyaA (Fig. 16). Moreover, IL-10 secretion was enhanced by both proteins. To further examine the effects of CyaA on CpG-ODN signalling, J774 macrophages and DC derived from C3H/HeN and BALB/c were incubated with A-CyaA or NA-CyaA in the presence of polymyxin B plus CpG-ODN. Both A-CyaA and NA-CyaA inhibited the production of TNF- and increased IL-10 production from CpG-ODN stimulated cells (data not shown). Taken together these findings demonstrate that the immunomodulatory effects of CyaA are not confined to, or dependant on, signalling of LPS through TLR-4, but can be mediated by the binding of other TLR ligands, such as CpG-ODN, to their receptors. Furthermore, these effects are not dependant on acylation of CyaA, but are enhanced by it.

The immunomodulatory effects are mediated through CD11b. To determine whether the cytokine modulatory effects of A-CyaA and NA-CyaA are mediated by their binding to cells via CD11b/CD18, cells were treated with the proteins and CpG-ODN in the presence of the anti-CD11b antibody M1/70, which has previously been

shown to block the binding of A-CyaA to host cells (14). Addition of the anti-CD11b antibody suppressed the ability of A-CyaA and NA-CyaA to inhibit CpG-ODN-induced production of TNF- and also their ability to synergise with CpG-ODN to enhance IL-10 secretion (Fig. 17). Similar results were obtained when CpG-ODN was replaced with LPS (data not shown). The enhancing effect of anti-CD11b on CpG-ODN-induced TNF-α (or LPS-induced TNF-α; data not shown) in the presence of CyaA or NA-CyaA probably reflects removal of the inhibitory effect of CyaA (which can no longer bind to its receptor), and retention of the effect of the LPS co-purified with the CyaA preparations. Thus modulation of macrophage cytokine release by A-CyaA and NA-CyaA involves interaction with the CD11b/CD18, suggesting specific uptake of both these proteins into innate immune cells through this cell surface receptor.

Influence of acylation on the modulation of DC maturation by CyaA. Maturation of DC in response to pathogen-derived molecules, such as LPS and CpG-ODN, can be detected by changes in cell surface expression of co-stimulatory molecules. Here we examined the effect of acylation of CyaA on its ability to induce maturation of immature bone-marrow-derived DC or to modulate LPS or CpG-ODN-induced maturation of DC. The effects of A-CyaA and its non-acylated derivative were tested in the presence of polymyxin B in order to examine their effects in the absence of TLR-4 signalling. Both NA-CyaA and A-CyaA enhanced DC surface expression of CD80 and suppressed endogenous CD40 expression in the presence of polymyxin B. Furthermore, pre-incubation of DC with NA-CyaA and A-CyaA suppressed the increase in surface expression of CD40 and ICAM-1 in response to LPS (Fig. 18). The modulatory effects were more pronounced with A-CyaA, supporting our hypothesis that acylation enhances the efficiency of CyaA-induced immunomodulatory effects. In order to confirm that CyaA also modulated responses to other TLR ligands, we examined its effects on CpG-ODN-induced maturation in DC. CpG-ODN enhanced surface expression of CD80 and CD40, whereas A-CyaA and NA-CyaA enhanced surface expression of CD80 but inhibited CpG-ODN-induced CD40 on DC from C3H/HeJ mice and on DC from BALB/c mice in the

presence of polymyxin B (data not shown). These data demonstrate that CyaA modulates DC maturation in response to TLR-4 and TLR-9 ligands and that acylation is not essential for these effects.

Acylation is not required for the adjuvant activity of CyaA. Having demonstrated that NA-CyaA and A-CyaA exert similar effects on innate cells *in vitro*, we examined the influence of acylation on the adjuvant activity of CyaA *in vivo*. Mice were immunised with KLH in the presence or absence of A-CyaA or NA-CyaA. Antigen-specific T-cell proliferation and cytokine production by draining popliteal lymph nodes were examined 7 days later. The results reveal that both proteins have comparable adjuvant activity. KLH-specific T cell proliferation and cytokine production were low or undetectable in lymph node cells from mice immunised with KLH alone (Fig. 19). However lymph node cells from these mice did proliferate and secrete IL-4, IL-5, IL-10 and IFN-γ in response to PMA and anti-CD3. In contrast, antigen-specific IL-4, IL-5 and IL-10 production (Fig. 19A) and proliferation (Fig. 19B) were detected in lymph node cells from mice immunised with KLH in the presence of A-CyaA or NA-CyaA. IFN- was produced by lymph node cells from mice immunised with KLH in the presence of A-CyaA or NA-CyaA (Fig. 19A), however, the concentrations were low when compared with that observed in mice immunised with KLH in the presence of CpG-ODN (unpublished observations). This cytokine profile is consistent with the data in Fig 1 showing that CyaA preferentially enhances Th2 and Tr1 cells. Analysis of antibody titres revealed that NA-CyaA and A-CyaA enhance IgG responses to KLH (Fig. 19C). IgG1 was enhanced to a greater extent than IgG2a, which is consistent with the selective enhancement of Th2 responses. Thus, lack of acylation does not compromise the adjuvant properties of CyaA or its ability to direct the induction of Th2 and Tr1 cells *in vivo*.

Murine model for multiple sclerosis

Experimental autoimmune encephalomyelitis (EAE) is a murine model for multiple sclerosis. EAE is induced in C57BL/6 mice by s.c. administration of 150 μg MOG peptide emulsified in complete Freund's adjuvant, supplemented with 1 mg

Mycobacteria tuberculosis intraperitoneal (i.p.) injection of 500 ng pertussis toxin, followed 2 days later by a second i.p. injection with 500 ng pertussis toxin. Mice develop symptoms of paralysis. In experiments to assess the effects of CyaA as an adjuvant for a vaccine against autoimmune disease, mice were immunized subcutaneously (s.c.) with 50 μg MOG peptide (residues 35-55) and 1.0 μg CyaA in phosphate buffered saline. This was repeated 21 days later. Control mice received MOG peptide or saline only. 7 days after the second immunization EAE was induced with MOG, Freund's adjuvant and pertussis toxin as described above. Mice were assessed daily for clinical signs of EAE, and scored as follows: 1 = tail paralysis, 2 = wobbly gait, 3 = hind limb weakness, 4 = hind limb paralysis, 5 = complete paralysis of hind and fore limbs, 6 = death.

The effect of immunization with myelin oligodendrocyte (MOG) peptides with CyaA on the disease progression (average disease index) in experimental autoimmune encephalomyelitis (EAE) is shown in Fig. 20. Fig. 21 shows the average disease score over time in an EAE model. Histology results clearly show the effect of immunisation with MOG and CyaA (Fig. 22).

Table 1 shows the disease score and disease index results. The results indicate that the administration of CyaA as an adjuvant significantly inhibits disease progression.

Immunization Group	Incidence	Day of onset	Mean Max. Clinical Score	Disease Index at day 23
Control	10/11	16.4	2.9	195
MOG	7/8	15	1.875	100
MOG + ACT	8/8	17.6	1.125	6.39

Table 1

Incidence is the number of mice out of the number tested that develop any clinical symptoms of EAE. The disease index was calculated by adding all daily average disease scores, dividing the average day of onset, and multiplying by 100.

Dosage, Mode of Administration and Pharmaceutical Formulations

The invention includes methods of modulating an immune response in a mammal to a selected antigen, the method comprises administering to a mammal a therapeutic amount of an agent comprising CyaA or a derivative or mutant or fragment or variant or peptide thereof or products of cells activated by these materials or administering a therapeutic amount of an agent comprising CyaA or derivative or mutant or fragment or variant or peptide thereof and an antigen or CyaA and a pharmaceutically acceptable toll-like receptor (TLR) ligand.

The compositions for administration may be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in or suspension in, liquid prior to infection can also be prepared. The preparation can also be emulsified, or the composition encapsulated in liposomes. The active immunogenic ingredients are often mixed with carriers which are pharmaceutically acceptable and compatible with the active ingredient. The term "pharmaceutically acceptable carrier" refers to a carrier that does not cause an allergic reaction or other untoward effect in subjects to whom it is administered. Suitable pharmaceutically acceptable carriers include, for example, one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the immunomodulator/formulation can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the formulation / immunomodulator.

Compositions of the invention may be administered parenterally, by injection, for example, either subcutaneously, epicutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories, and in some cases, oral formulations, nasal formulations or formulations suitable for distribution as aerosols. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides;

such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25-70%.

The compositions of the invention may be formulated into the immunomodulator compositions as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or with organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The composition may be administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The quantity to be administered depends on the subject to be treated, including, e.g., capacity of the subject's immune system to synthesize anti-inflammatory cytokines or to induce regulatory T cells, and the degree of protection desired. Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 0.1 μg/g to 1000 μg/g, such as in the range from about 0.1 μg to 100 mg. Suitable regimens for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and may be peculiar to each subject.

It will be apparent to those of skill in the art that the therapeutically effective amount of the CyaA composition will depend, inter alia, upon the administration schedule, the unit/ dose of antigen administered, whether the CyaA is administered in combination with other therapeutic agents, the immune status and health of the recipient, and the therapeutic activity of the particular CyaA /antigen complex.

The composition may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of administration can include 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the effect on the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. Periodic administration at intervals of 1-5 years, usually 3 years, are desirable to maintain the desired levels of protection.

A series of vaccinations may be given, for example, at intervals of 3 months, or of four months, or of six months, between inoculations. Such a series may include, for example, 3 or 4 or 5 vaccinations in total. For vaccinations given to infants, a series of vaccination may be given, e.g., at birth or within the first week, and then at 6, 10 and 14 weeks of life. A series of vaccinations may be given at birth, and at 1, 3 and 6 months of life.

The composition may be administered for therapeutic use a number of times per week such as twice per week, weekly, a number of times per month, monthly for a number of weeks or months, for a year or for several years. The composition for therapeutic use may comprise the active ingredient on its own or in combination with a self-antigen. The therapy may also involve administration of other drugs either at the same time (either in the same formulation or separately) or at spaced time intervals.

A therapeutically effective dose may vary depending upon the route of administration and dosage form. Specific dosages may be adjusted depending on

conditions of disease, the age, body weight, general health conditions, sex, diet of the subject, dose intervals, administration routes, excretion rate, and combinations of drugs. Any of the dosage forms containing effective amounts are well within the limits of routine experimentation. The compositions of the invention may also be administered in conjunction with other drugs including those used in the treatment of autoimmune disease. The compositions may also be administered alone using a similar dosage regime as used for other treatments of autoimmune disorders. The term "treatment" is intended to include an alleviation of symptoms associated with a disorder or disease, or the halt of further progression or worsening of those symptoms, or prevention or prophylaxis of the disease or disorder.

The course of the treatment can be followed by testing ex vivo cytokine production by cells of the immune system (recovered from blood samples) with and without in vitro stimulation with for example LPS. The assays can be performed using conventional reagents for culture of cells and quantification of cytokines using antibodies and the like. These techniques are commonly known to one skilled in the art.

The invention is not limited to the embodiments hereinbefore described which may be varied in detail.

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